EXHIBIT

Serial No. 09/778,187   2873-US

HuB7L1-CoR Full Length

(Linear) (Six Base) MAP of: 4469-Wi26.Seq check: 1995 from: 1 to: 1535
[hollingsworth.cncdna.4469]
req 4469 HuB7L1 counterstructure Wi26 pool314-28#34 FINAL SEQUENCE FILE
3mGel1648, #7046, #5080 / 3mGel1663 dpc7266,67 /2mGel1671 dpc7305,6
4469-wi26

```
        B                                                    B
        ENXs                       B                        :aX
        aomi                       c                        .mh
        etaE                       g                         Ho
        1131                       l                         12
        //               Sal-22778 →                        /
        GCGGCCGCGCCCGACATGGCGAGTGTAGTGCTGCCGAGCGGATCCCAGTGTGCGGCGGCA
   1 ---------+---------+---------+---------+---------+---------+ 60
        CGCCGGCGCGGGCTGTACCGCTCACATCACGACGGCTCGCCTAGGGTCACACGCCGCCGT
   a                  M  A  S  V  V  L  P  S  G  S  Q  C  A  A  A  -


                                           B
                                           s
        N                B      B          p
        s                BsKNH  AsBSX      B1                      ES    D
        P                aaaaa  vrgmm      a2                      aa    s
        B                nHsre  aFlaa      n8                      rp    a
        2                11112  11111      26                      11    1
                         //     / //       /                        /
        GCGGCGGCGGCGGCGCCTCCCGGGCTCCGGCTCCGGCTTCTGCTGTTGCTCTTCTCCGCC
  61 ---------+---------+---------+---------+---------+---------+ 120
        CGCCGCCGCCGCCGCGGAGGGCCCGAGGCCGAGGCCGAAGACGACAACGAGAAGAGGCGG
   a  A  A  A  A  A  P  P  G  L  R  L  R  L  L  L  L  L  F  S  A  -


        N                                    A
        sS                                   l
        ps                                   w
        Bt                                   N
        22                                   1
        GCGGCACTGATCCCCACAGGTGATGGGCAGAATCTGTTTACGAAAGACGTGACAGTGATC
 121 ---------+---------+---------+---------+---------+---------+ 180
        CGCCGTGACTAGGGGTGTCCACTACCCGTCTTAGACAAATGCTTTCTGCACTGTCACTAG
   a   A  A  L  I  P  T  G  D  G  Q  N  L  F  T  K  D  V  T  V  I  -
     Signal seq.  ^
        GAGGGAGAGGTTGCGACCATCAGTTGCCAAGTCAATAAGAGTGACGACTCTGTGATTCAG
 181 ---------+---------+---------+---------+---------+---------+ 240
        CTCCCTCTCCAACGCTGGTAGTCAACGGTTCAGTTATTCTCACTGCTGAGACACTAAGTC
   a   E  G  E  V  A  T  I  S  C  Q  V  N  K  S  D  D  S  V  I  Q  -


                        A                  E
                        l                  c                         B
                        w                  o               S         s
                        N                  5               t         p
                        1                  7               u         M
```

```
           1                     1                  1    1
         CTACTGAATCCCAACAGGCAGACCATTTATTTCAGGGACTTCAGGCCTTTGAAGGACAGC
     241 ---------+---------+---------+---------+---------+---------+ 300
         GATGACTTAGGGTTGTCCGTCTGGTAAATAAAGTCCCTGAAGTCCGGAAACTTCCTGTCG
    a     L  L  N  P  N  R  Q  T  I  Y  F  R  D  F  R  P  L  K  D  S  -


                 A
                 l   A                                          P      B
                 w   p                                          s      s
                 N   o                                          h      m
                 l   l                                          A      B
                                                                1      1
         #30518 (7A) →
         AGGTTTCAGTTGCTGAATTTTTCTAGCAGTGAACTCAAAGTATCATTGACAAACGTCTCA
     301 ---------+---------+---------+---------+---------+---------+ 360
         TCCAAAGTCAACGACTTAAAAAGATCGTCACTTGAGTTTCATAGTAACTGTTTGCAGAGT
    a     R  F  Q  L  N  F  S  S  S  E  L  K  V  S  L  T  N  V  S  -


         #30509 (1A/6A) →                                    #30516
         ATTTCTGATGAAGGAAGATACTTTTGCCAGCTCTATACCGATCCCCCACAGGAAAGTTAC
     361 ---------+---------+---------+---------+---------+---------+ 420
         TAAAGACTACTTCCTTCTATGAAAACGGTCGAGATATGGCTAGGGGGTGTCCTTTCAATG
    a     I  S  D  E  G  R  Y  F  C  Q  L  Y  T  D  P  P  Q  E  S  Y  -


                               B                    B  E
                 X             s                    C  s  c
                 c             a                    l  a  o
                 m             A                    a  B  R
         (5A) →   l                                 1  1  S
         ACCACCATCACAGTCCTGGTCCCACCACGTAATCTGATGATCGATATCCAGAAAGACACT
     421 ---------+---------+---------+---------+---------+---------+ 480
         TGGTGGTAGTGTCAGGACCAGGGTGGTGCATTAGACTACTAGCTATAGGTCTTTCTGTGA
    a     T  T  I  T  V  L  V  P  P  R  N  L  M  I  D  I  Q  K  D  T  -


                                  H
                 B                i                  E  B
                 s                n                  a  a
                 g                c                  e  l
                 l                2                  1  1
         #30514 (4A) →
         GCGGTGGAAGGTGAGGAGATTGAAGTCAACTGCACTGCTATGGCCAGCAAGCCAGCCACG
     481 ---------+---------+---------+---------+---------+---------+ 540
         CGCCACCTTCCACTCCTCTAACTTCAGTTGACGTGACGATACCGGTCGTTCGGTCGGTGC
    a     A  V  E  G  E  E  I  E  V  N  C  T  A  M  A  S  K  P  A  T  -
                                                    E
                                                    a
                                                    r
                                                    1
         ACTATCAGGTGGTTCAAAGGGAACACAGAGCTAAAAGGCAAATCGGAGGTGGAAGAGTGG
     541 ---------+---------+---------+---------+---------+---------+ 600
         TGATAGTCCACCAAGTTTCCCTTGTGTCTCGATTTTCCGTTTAGCCTCCACCTTCTCACC
      ← #30517 (5B/6B/7B)
```

```
a         T  I  R  W  F  K  G  N  T  E  L  K  G  K  S  E  V  E  E  W  -
                                                          B
                                                          s
                  N                       N           A   pH         E
            A     s              sP           p   1g         c
            f     p              pv           a   2i         o   D D
            l     H              Bu           L   8A         5   r r
            3     1              22           1   61         1   1 2
          TCAGACATGTACACTGTGACCAGTCAGCTGATGCTGAAGGTGCACAAGGAGGACGATGGG
    601   ---------+---------+---------+---------+---------+---------+  660
          AGTCTGTACATGTGACACTGGTCAGTCGACTACGACTTCCACGTGTTCCTCCTGCTACCC
a         S  D  M  Y  T  V  T  S  Q  L  M  L  K  V  H  K  E  D  D  G  -


                                          B
                                          s
            P                             pH                      B   N
            p  P                           1g           S   P     s   s
            u  s                           2i           f   s     p   p
            M  s                           8A           c   t     M   B
            1  1                           61           1   1     1   2
          GTCCCAGTGATCTGCCAGGTGGAGCACCCTGCGGTCACTGGAAACCTGCAGACCCAGCGG
    661   ---------+---------+---------+---------+---------+---------+  720
          CAGGGTCACTAGACGGTCCACCTCGTGGGACGCCAGTGACCTTTGGACGTCTGGGTCGCC
a         V  P  V  I  C  Q  V  E  H  P  A  V  T  G  N  L  Q  T  Q  R  -


                                          B
                                          s
                               A          pH                  E
            X                  S   p  1g                       c
            b                  m   a  2i                       o
            a                  1   L  8A                       N
            1                  1   1  61                       1
          TATCTAGAAGTACAGTATAAGCCTCAAGTGCACATTCAGATGACTTATCCTCTACAAGGC
    721   ---------+---------+---------+---------+---------+---------+  780
          ATAGATCTTCATGTCATATTCGGAGTTCACGTGTAAGTCTACTGAATAGGAGATGTTCCG
                                          ← #30515 (4B)
a         Y  L  E  V  Q  Y  K  P  Q  V  H  I  Q  M  T  Y  P  L  Q  G  -

              B                           H   N
            AsSX                 S        iHA  s
            vrmm                 m        npf  p
            aPaa                 1        cal  H
            1111                 1        213  1
          TTAACCCGGGAAGGGGACGCGCTTGAGTTAACATGTGAAGCCATCGGGAAGCCCCAGCCT
    781   ---------+---------+---------+---------+---------+---------+  840
          AATTGGGCCCTTCCCCTGCGCGAACTCAATTGTACACTTCGGTAGCCCTTCGGGGTCGGA
```

```
a        L   T   R   E   G   D   A   L   E   L   T   C   E   A   I   G   K   P   Q   P   -

           GTGATGGTAACTTGGGTGAGAGTCGATGATGAAATGCCTCAACACGCCGTACTGTCTGGG
     841   --------+---------+---------+---------+---------+---------+ 900
           CACTACCATTGAACCCACTCTCAGCTACTACTTTACGGAGTTGTGCGGCATGACAGACCC
a        V   M   V   T   W   V   R   V   D   D   E   M   P   Q   H   A   V   L   S   G   -


                 B                                                  
                 s                                                  
                 p                                          N       H
                 AB1                                        s       i
                 pa2                                        p       n
                 an8                                        B       d
                 126                                        2       3
                 //
           CCCAACCTGTTCATCAATAACCTAAACAAAACAGATAATGGTACATACCGCTGTGAAGCT
     901   --------+---------+---------+---------+---------+---------+ 960
           GGGTTGGACAAGTAGTTATTGGATTTGTTTTGTCTATTACCATGTATGGCGACACTTCGA-
                                              (-ggatatcactcagcataatgtata t7 Promoter)
a        P   N   L   F   I   N   N   L   N   K   T   D   N   G   T   Y   R   C   E   A   -

                                                      41-mer  33713


                                                    B
                                                    s
                                                    t
                                       T            AZ
                                       t            c1
                                       h            c7
           #30511 (2A/3A) →           3            11
           TCAAACATAGTGGGGAAAGCTCACTCGGATTATATGCTGTATGTATACGATCCCCCCACA
     961   --------+---------+---------+---------+---------+---------+ 1020
           AGTTTGTATCACCCCTTTCGAGTGAGCCTAATATACGACATACATATGCTAGGGGGGTGT
a        S   N   I   V   G   K   A   H   S   D   Y   M   L   Y   V   Y   D   P   P   T   -

           ACTATCCCTCCTCCCACAACAACCACCACCACCACCACCACCACCACCACCACCATCCTT
    1021   --------+---------+---------+---------+---------+---------+ 1080
           TGATAGGGAGGAGGGTGTTGTTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTAGGAA
                                                            ← #30512 (2B)
a        T   I   P   P   P   T   T   T   T   T   T   T   T   T   T   T   T   T   I   L   -

           B
           s        A
           p        v
           M        a
           1        l                                            start T.M.
           ACCATCATCACAGATTCCCGAGCAGGTGAAGAAGGCTCGATCAGGGCAGTGGATCATGCC
    1081   --------+---------+---------+---------+---------+---------+ 1140
           TGGTAGTAGTGTCTAAGGGCTCGTCCACTTCTTCCGAGCTAGTCCCGTCACCTAGTACGG
           ←                        ← #30513 (3B)
```

```
a      T  I  I  T  D  S  R  A  G  E  E  G  S  I  R  A  V  D  H  A   -

                B                          T
                s                          t
                a                          h
                H                          3
                1                          2
       GTGATCGGTGGCGTCGTGGCGGTGGTGGTGTTCGCCATGCTGTGCTTGCTCATCATTCTG
  1141 --------+---------+---------+---------+---------+---------+ 1200
       CACTAGCCACCGCAGCACCGCCACCACCACAAGCGGTACGACACGAACGAGTAGTAAGAC
a      V  I  G  G  V  A  V  V  V  F  A  M  L  C  L  L  I  I  L   -

                H                          B
                a                          s
                e                          P
                2                          H
                                           1
       GGGCGCTATTTTGCCAGACATAAAGGTACATACTTCACTCATGAAGCCAAAGGAGCCGAT
  1201 --------+---------+---------+---------+---------+---------+ 1260
       CCCGCGATAAAACGGTCTGTATTTCCATGTATGAAGTGAGTACTTCGGTTTCCTCGGCTA
a      G  R  Y  F  A  R  H  K  G  T  Y  F  T  H  E  A  K  G  A  D  -

       GACGCAGCAGACGCAGACACAGCTATAATCAATGCAGAAGGAGGACAGAACAACTCCGAA
  1261 --------+---------+---------+---------+---------+---------+ 1320
       CTGCGTCGTCTGCGTCTGTGTCGATATTAGTTACGTCTTCCTCCTGTCTTGTTGAGGCTT
                                                  ← #30510 (1B)
a      D  A  A  D  A  D  T  A  I  I  N  A  E  G  G  Q  N  N  S  E  -

                S                X
                c                b
                a                a
                1                l
       GAAAAGAAAGAGTACTTCATCTAGATCAGCCTTTTTGTTTCAATGAGGTGTCCAACTGGC
  1321 --------+---------+---------+---------+---------+---------+ 1380
       CTTTTCTTTCTCATGAAGTAGATCTAGTCGGAAAAACAAAGTTACTCCACAGGTTGACCG
a      E  K  K  E  Y  F  I  *

                                           A
                                           p
                                           o
                                           1
       CCTATTTAGATGATAAAGAGACAGTGATATTGGAACTTGCGAGAAATTCGTGTGTTTTT
  1381 --------+---------+---------+---------+---------+---------+ 1410
       GGATAAATCTACTATTTCTCTGTCACTATAACCTTGAACGCTCTTTAAGCACACAAAAAA

       TATGAATGGGTGGAAAGGTGTGAGACTGGGAAGGCTTGGGATTTGCTGTGTAAAAAAAAA
  1441 --------+---------+---------+---------+---------+---------+ 1500
       ATACTTACCCACCTTTCCACACTCTGACCCTTCCGAACCCTAAACGACACATTTTTTTT
                                    B
                                  ENKs
                                  aomi
                                  etaE
```

```
                                                1131
                                                 //
              AAAAAAATGTTCTTTGGAAAGAAAAAAGCGGCCGC
     1501  ---------+---------+---------+-----  1535
              TTTTTTTACAAGAAACCTTTCTTTTTTTCGCCGGCG
```

Enzymes that do cut:

Acc1	Afl3	AlwN1	Apo1	Apa1	ApaL1	Ava1	Ball
BamH1	Ban1	Ban2	Bcg1	Bgl1	BsaA1	BsaB1	BsaH1
Bsg1	BsiE1	BsmB1	Bsp1286	BspH1	BspM1	BsrF1	BstZ171
Cla1	Dra2	Drd1	Dsa1	Eae1	Ear1	Eco571	EcoN1
EcoR5	Hae2	HgiA1	Hinc2	Hind3	Hpa1	Kas1	Nar1
Not1	NspB2	NspH1	PpuM1	PshA1	Pss1	Pst1	Pvu2
Sap1	Sca1	Sfc1	Sma1	Sml1	Sst2	Stu1	Tth32
Xba1	Xcm1	Xho2	Xma1	Xma3			

Enzymes that do not cut:

Aat2	Acl1	Afl2	Age1	Asc1	Ase1	Asp718	Asu2
Avr2	Bbs1	BciV1	Bcl1	Bgl2	Bpu11021	Bpm1	Bsa1
BsiW1	Bsm1	BspE1	BssH2	BstE2	BstX1	Bsu361	Dra1
Dra3	Eam1105	Eco473	EcoR1	Fse1	Fsp1	Kpn1	Mlu1
Mun1	Nco1	Nde1	NgoM1	Nhe1	Nru1	Nsi1	Pac1
Pf1M1	Pme1	Pml1	Pvu1	Rsr2	Sal1	Sfi1	SgrA1
SnaB1	Spe1	Sph1	Srf1	Sse8387	Ssp1	Sst1	Sty1
Swa1	Tth31	Xho1	Xmn1				

REVIEW

Approaches to DNA Mutagenesis: An Overview

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Mutagenesis of proteins is of paramount importance for understanding the relationship of protein structure to function. The functional and structural roles of amino acid residues in a protein of interest can be studied by comparing the mutant protein carrying changes in amino acid residues to the wild-type protein. To obtain samples of a specific mutant protein, the mutant gene must be isolated or created. Before the era of site-directed mutagenesis (SDM),[2] the only way to obtain a mutation was to isolate naturally occurring mutants with phenotypic or selective screening. Because the rate of naturally occurring errors in DNA synthesis is very low, such screening was usually done after treatment with mutagenic agents. Moreover, even if a screening test was available, isolation of mutants that are lethal or that do not produce observable changes in phenotype was not possible (1). In this procedure, also, the position and the type of mutations in the mutants obtained were often distributed randomly in the gene of interest, which may not have been what the researcher desired.

Mutagenesis, now achieved by either polymerase

[2] Abbreviations used: SDM, site-directed mutagenesis; REM, random and extensive mutagenesis; PCR, polymerase chain reaction; UDG, uracil DNA N-glycosylase; DMSO, dimethyl sulfoxide; LCR, ligase chain reaction; USE, unique site elimination; bp, base pair(s); Taq-Taq, Pfu-Pfu, and Vent-Vent, DNA polymerases; dI, deoxyinosine phosphoramidite, monophosphate; dITP-deoxyinosine triphosphate; dATP, dCTP, dGTP, or dTTP-deoxyadenosine, deoxycytosine, deoxyguanosine, or deoxythymine triphosphate; dNTP-dATP, dCTP, dGTP, and/or dTTP.
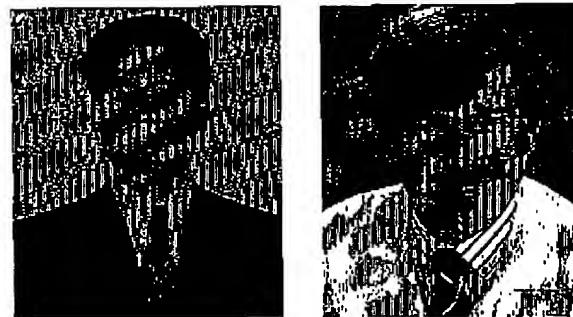
chain reaction (PCR) or non-PCR, has revolutionized the means by which mutants are obtained (2, 3). Mutations can now be created precisely at a specific residue with a specific codon change to produce the desired amino acid substitution (SDM), which has allowed alteration of any amino acid residue in a protein without extensive screening. Thus, the SDM approach is much more efficient, yielding desired mutations in 50–100% of the molecules produced, than that of phenotypic screening (<1%). When a set of random mutations over a region or the entirety of a gene is desired, the approach of random and extensive mutagenesis (REM, also known as targeted random, region-specific, or library mutagenesis) has been developed, and has been improved greatly in recent years. The introduction of PCR has made both DNA synthesis and DNA mutagenesis very efficient processes, compared to that using the thermolabile polymerase and the single-stranded DNA as the template (non-PCR).

In recent years, advances in mutagenesis methods have made mutagenesis technology on the whole exceedingly complex. Here we review some of the newer approaches to *in vitro* DNA mutagenesis methods in both the PCR and the non-PCR categories. In addition, some of the *in vivo* DNA mutagenesis methods are briefly described. The applications of these methods in SDM, REM, scanning mutation, and deletion have been discussed. This review is intended to guide investigators, novice and experienced alike, in selecting DNA mutagenesis protocols according to their needs.

BASIC PROCEDURES OF DNA MUTAGENESIS

To mutagenize a gene is to synthesize enzymatically a new DNA, while in the meantime incorporating the

157

158   LING AND ROBINSON

desired mutations into this newly synthesized DNA. Other than attempting to introduce mutations into the product, the methods or protocols for DNA mutagenesis are essentially the same as those for DNA synthesis or for other molecular biological manipulations, such as cloning, sequencing, and probe labeling.

Three essential components are required in an *in vitro* DNA synthesis reaction: the template, the primer, and the four dNTPs, i.e., dATP, dCTP, dGTP, dTTP, in the presence of the enzyme, the DNA polymerase. In *in vitro* DNA synthesis, as *in vivo*, the DNA is semiconservatively synthesized as a single strand, incorporating the building blocks, dNTPs, based on base-pairing, i.e., G with C and A with T, with the template strand. *In vitro*, a short single-stranded DNA, referred to as a primer, is always required to be annealed to the template strand DNA to initiate the new DNA strand synthesis. Thus, dNTPs are always added, base by base, to the 3'-end of the primer; thus, *in vitro* DNA synthesis is often referred to as the primer extension. The template DNA, whether double-stranded or single-stranded, provides the existing strand to synthesize a second, new strand of DNA. For this reason, the product of DNA synthesis by the enzymatic reaction is always double-stranded DNA, made of the original (template) strand and the new (product) strand. Buffers and various other conditions are also important to ensure the success and the fidelity of DNA synthesis.

There are two types of *in vitro* DNA synthesis modules: the thermostable polymerase-based PCR and the thermolabile polymerase-based non-PCR. Accordingly, the DNA mutagenesis methods can be classified into the thermostable polymerase PCR and the thermolabile polymerase non-PCR, in addition to the *in vivo* DNA mutagenesis methods.

Thermostable DNA Polymerase-Based PCR

PCR involves *in vitro* synthesis of a DNA product, the two ends of which are defined by the positions of the two designed primers. As described above, DNA synthesis *in vitro* always requires an oligonucleotide primer, usually 20-30 bp in length, complementary to the template. These primers can be synthesized chemically exactly according to the designed sequence. Since a small number of mismatches, e.g., 1-3 bp, are tolerable for primer binding to the template, mismatches can be incorporated as mutations precisely in the newly synthesized DNA. Other than mismatches introduced through the primer, the mutant DNA is reproduced exactly according to the wild-type DNA template. Because of its simplicity, the primer has become a convenient source for introducing mutations (for reviews, see 2, 3, 100). PCR undergoes multiple heating and/or cooling cycles (average 30 cycles), each having three phases: denaturation at about 95°C, annealing at about 55°C, and extension at about 72°C. At the denaturation phase, the template is denatured so that the two strands are separated, to allow the primers to bind or anneal to them. At the annealing phase, the two primers are annealed to one strand each. At the extension phase, the annealed primers are then extended according to the template strands. Many cycles are then repeated in a such way. Thus both the original templates and their products, which predominate after several cycles over the original templates, become further templates for subsequent rounds of DNA synthesis. In this way, templates are amplified exponentially by around 2^{20}-fold (13) to yield linear double-stranded mutant PCR products. PCR DNA synthesis is thus often called the amplification of the template DNA. Since thermostable DNA polymerases (usually *Taq* DNA polymerase) remain active throughout the high-temperature cycling, they need only be added once, at the beginning of the multiple cycles.

Usually, a mutant gene fragment or an entire mutant gene is produced by the PCR. A mutant fragment can then be used to replace the wild-type gene fragment in a plasmid or an entire mutant gene can be subcloned into a vector, for mutant screening and characterization. Thus, most often the subcloning is required, except that in the case of the inverse PCR, an entire linear mutant plasmid can be produced and then simply can be circularized.

Templates

The template DNA used for the PCR can be double-stranded or single-stranded, circular or linear. For the single-stranded template, in the first cycle only one of the two primers binds to this single strand and gets extended to form the double stranded-DNA template for the subsequent cycles. However, double-stranded plasmid DNAs have become favored for thermostable polymerase-based PCR DNA synthesis for several reasons. In comparison to single-stranded DNAs, double-stranded DNAs are easier to prepare. Also, gene inserts are in general more stable with double-stranded DNAs. Mutagenesis efficiency (50-100%) is similar for double-stranded and single-stranded templates. This trend has been reflected by the fact that the most recent commercial kits are based on double-stranded templates (Table 1).

Primer/Template Annealing

To ensure successful DNA mutagenesis, general rules for designing a good primer should be followed. Particularly, a primer for DNA synthesis should be free of strong secondary structures, such as hairpins, stem-loops, or direct repeats. Mismatches in general should be placed in the middle of a primer rather than at the 5'-terminus, although with some methods, such as the

APPROACHES TO DNA MUTAGENESIS: AN OVERVIEW   159

TABLE 1

A Survey of Available Commercial Kits for SDM

Company, kit	Year	Method	Reference
		I. Kits for single-stranded templates	
Bio-Rad, MutaGene II	1991	dUTPase selection	(148)
USB, T7-GEN	1991	Methyl-dCTP	(147)
Amersham, Sculptor	1994	Phosphorothioate analogue	(146)
		II. Kits for double-stranded templates	
Stratagene, DoubleTake	1992	Solid-phase strand separation	(48)
Clontech, Transformer	1994	Unique site elimination	(11)
GIBCO-BRL, PCR SDM	1994	PCR and UDG cloning	(23)
New England Biolabs, Code20	1994	Unique site near the mutagenesis site	(174)
Pharmacia Biotech, USE	1994	Unique site elimination	(11)
Promega, Altered Sites II	1994	Antibiotic resistance repairment	(140)
Stratagene, Chameleon	1994	Unique site elimination	(11)
Stratagene, ExSite	1995	Inverse PCR	(90)

inverse PCR and the ligation PCR (will be described later), it is preferable to place the mutation at the 5'-termini of the primers. Mismatches at the 3'-terminus of a primer generally affect the rates of DNA synthesis, although exceptions have been reported (77).

To hybridize the mutagenic primer correctly and sufficiently to its template, the annealing temperature should be chosen carefully. In general, the annealing temperature is chosen to be a few degrees below the melting temperature (T_m, the temperature at which half of the oligonucleotides are annealed to templates) of an oligonucleotide. Without any mismatches, the melting temperature in degrees Celsius is estimated (98) simply as follows: $T_m = 4 \times$ (number of G and C bases) + $2\times$ (number of A and T bases). With mismatches, the annealing temperature should be reduced further according to the number of mismatches in the primer. Alternatively, the minimal magnesium concentration should be increased from 1.5 mM for a perfect match to 2.0 mM for 1 mismatch in a 23-bp oligonucleotide (1/23 mismatch length) (99). When a mismatch is 2 bp in a 24-bp oligonucleotide, Mg concentration should be increased to 3 mM; for a 9-bp mismatch in a 41-bp oligonucleotide, it should be increased to 4 mM.

Areas of Concern on PCR with Recommended Resolutions

PCR was applied to DNA mutagenesis almost immediately after its inception in 1985, and has become a very useful method for DNA mutagenesis. Several areas of concern, together with suggested methods of resolution, are discussed below.

Increasing the sequence fidelity of PCR products. Similar to the DNA synthesis by PCR, the main draw-

back of using PCR for DNA mutagenesis is the relatively high rate of sequence errors in PCR products, often creating undesired mutations in addition to intended ones. *Taq*, the most widely used thermostable polymerase, lacks the 3'-5' exonuclease activity that "proofreads" any errors caused by 5'-3' DNA polymerase during DNA synthesis. Amplification through many cycles therefore accumulates errors. Two other polymerases, *Pfu* and *Vent*, which carry 3'-5' exonuclease activity, have been shown to provide 6–15 times the sequence fidelity of *Taq* (14–16). Thus, since their introduction, *Pfu* and *Vent* have been preferred for PCR-based DNA site-directed mutagenesis (17–19). When *Taq* was combined with even a small amount of *Pfu* or *Vent*, the fidelity increased about twofold, presumably because of the introduction of proofreading ability (16, 20, 21).

The concentrations of magnesium ions, dNTP, and pH may also affect sequence fidelity (16). The number of cycles can be reduced to achieve high fidelity, although this decreases the final amount of PCR products. This paradox can be solved by using larger amounts of methylated templates, which are readily removed after PCR by digestion with *Dpn*I, a frequent-cutting used restriction enzyme which cleaves only methylated DNAs (22–25). Thus the use of a large amount of template makes it possible to amplify a large amount of products in a small number of cycles.

Increasing the size and yield of PCR products. Until recently, only products smaller than 3 kb could be amplified routinely by PCR. Longer products are often needed in experiments involving DNA mutagenesis (24). To obtain long PCR products, several factors must be optimized. For example, shorter denaturation time,

smaller reaction volume, longer extension time, and different buffer compositions (e.g., high pH, additional denaturants) are often used (26–29).

More recently, a small amount of 3'-5' exonuclease (*Pfu* or *Vent*), used as a secondary enzyme in addition to *Taq* as the main enzyme, has been shown to increase the size of PCR products (20). This improvement is attributed to the ability of 3'-5' exonuclease to correct *Taq*-induced sequence errors, which are thought to stall further amplification of large products (20). On the other hand, when *Pfu* or *Vent* was used alone without *Taq*, neither could support the amplification of long PCR products (20, 30). This may be the result of their 3'-5' exonuclease activity being higher than their 5'-3' polymerase activity. Therefore, the use of *Taq* and *Pfu* in the optimal combination is ideal for producing large mutant DNAs (19, 31). Mammalian genomic DNAs as large as 22 kb and phage λ-DNAs as large as 42 kb have been amplified successfully with this improved technique (20, 29). Kits for long PCR products are now commercially available from several sources.

Amplifying DNAs with secondary structures or GC-rich regions. Problems with secondary structures and GC-rich regions in DNA templates usually are remedied effectively with the use of PCR, which uses temperatures higher than those used in the thermolabile polymerase method, performed at 37°C. However, sometimes strong secondary structures or high GC-rich regions can remain in template DNAs even at temperatures employed in a PCR. Relevant to this problem, alkaline and heat denaturation before PCR can significantly improve the amplification of these DNAs (32, 33). "Hot PCR," in which the routine annealing temperature is increased from 55 to 65–72°C, is also useful in overcoming impediments caused by secondary structures or GC-rich regions (31, 34). In addition, denaturants (1–20% DMSO, 5–15% glycerol, 5–20% formamide, Tween-20, and NP-40) (34–38), T4 gene 32 protein (38), and *Escherichia coli* single-stranded DNA-binding protein (7) are helpful in amplifying such difficult DNAs. Similarly, tetramethylammonium chloride at 0.01–0.1 mM (39) or 10–120 mM (40) is effective in facilitating the amplification of difficult DNAs. Moreover, nucleotide analogs, 7-deaza-2'-deoxyguanosine triphosphate (41) or deoxyinosine triphosphate (42), can be incorporated into secondary structures or GC-rich regions to weaken the bonding between G and C bases.

Longer extension time is also beneficial for amplification of difficult DNAs (43). Presumably, the rate of DNA synthesis is lower at GC-rich regions or secondary structures. Finally, *Vent* and *Pfu* seem to be more capable of amplifying DNAs containing GC-rich regions or secondary structures than *Taq* (37, 43 and our own observation), although the mechanism is not clear.

Thermolabile Polymerase-Based Non-PCR

In thermolabile DNA polymerase-based DNA synthesis, the DNA template is denatured with alkali and/or heat and annealed with the primer at the room temperature. Usually, one primer is used in the thermolabile polymerase-based non-PCR methods and mutations are introduced through mismatches in the mutagenic primer. DNA templates can be single-stranded or double-stranded, although single-stranded templates are preferred in this method. In the case of double-stranded DNA, only one strand bound with the primer is used as the template strand and the other strand is not used at all. DNA synthesis is then carried out at 37°C with thermolabile polymerases, which would become inactivated at higher temperatures and thus are called thermolabile enzymes. Nucleotide bases are added to the 3'-end of the primer according to base-pairing with the template. Consequently, a single-stranded DNA, still linear, complementary to the template strand, circular, is produced (2). This product is then circularized. Thus the product of this type of DNA synthesis is a hybrid DNA duplex carrying one template strand and one newly synthesized mutant strand. This hybrid is then used to transform (for plasmid or phagemid hybrids) or infect (for phage hybrids) *E. coli* to segregate mutant DNAs from wild-type DNAs. If M13 phage mutants are obtained, they are subcloned into a double-stranded expression vector for protein studies. Because there is only one round of DNA synthesis, there is no amplification of the template. In addition, when denatured double-stranded DNA is used as the template, only one of the two strands is actually used for the synthesis of the mutant strand.

Templates

With thermolabile polymerases, mutant DNAs are synthesized more readily with a single-stranded than a double-stranded template, since two denatured complementary template strands of DNAs may quickly reanneal at 37°C, interfering with the primer annealing with the template strand.

To prepare single-stranded DNAs as templates, often a gene is cloned into an M13 phage vector or a phagemid vector, which contains an M13 or a f1 replication origin, respectively (44). This preparation is time-consuming. Also, larger DNA gene inserts tend to be spontaneously deleted from the M13 phage vector during the process of the preparation (45), although long inserts in this type of vectors have also been successfully mutagenized. The single-stranded DNAs obtained are then used as templates to synthesize a mutant strand, directed by a mutagenic primer.

Alternatively, single-stranded linear DNA templates can be prepared from linear double-stranded plasmid DNAs or from PCR products that are always linear (46).

The two strands are separated from each other by the biotinylation of one strand (47). This biotinylated single-stranded DNA can then be used as the template for mutagenesis. For example, Weiner and colleagues (48) used such a template to synthesize a portion of a new strand with a mutagenic primer; the remaining portion was synthesized with an upstream primer. These two portions were then ligated. The resulting complete linear mutant strand was then separated from the biotinylated wild-type strand and recircularized. This circularization was aided by a bridging oligonucleotide binding to the two ends. The second mutant strand was then used to form the double-stranded mutant DNA. Although the steps are multiple, this approach avoids the problems or inconvenience of M13 phage or phagemid vectors. A kit is also available (Table 1).

Single-stranded linear DNAs can also be obtained from double-stranded DNAs by an asymmetric PCR—a PCR in which only one primer, rather than two, is used; thus, only one strand is linearly amplified. Sometimes, one primer is used at a concentration much higher than that of the other primer to produce mostly asymmetric products along with a small amount of double-stranded products. This type of template has allowed mutagenesis of relatively large mutant DNAs (0.5 and 1.1 kb) (49, 50).

Thermolabile Enzymes

Escherichia coli DNA polymerase, a 5'-3' polymerase, was the enzyme first used in the thermolabile polymerase-based DNA synthesis. However, this polymerase is also active in other ways, having 5'-3' exonuclease activity and strand-displacement (helicase) activity as well. These two additional activities are undesirable in DNA mutagenesis procedures: mismatches introduced by the mutagenic primer can be removed by 5'-3' exonuclease activity, and newly synthesized DNA containing mutations can be displaced by wild-type DNAs by strand-displacement activity. Klenow, a proteolytic fragment of *E. coli* DNA polymerase, is more efficient for DNA mutagenesis, since it does not possess 5'-3' exonuclease activity; nonetheless, it does retain strand-displacement activity.

In recent years, phage-derived enzymes, i.e., T4 DNA polymerase, T7 DNA polymerase and sequenases (derived from T7 DNA polymerase without the native 3'-5' exonuclease activity), have become the most frequently used enzymes for DNA mutagenesis, since they do not carry either extra activity.

Primer/Template Annealing

The general rules for the designing of primers mentioned in the PCR section should be followed. In addition, the non-PCR method requires a good region of match at each end, usually 10–15 bases. When the 5'-

terminus of a primer has a strong anchorage to its template, the strand displacement caused by Klenow or other enzymes will be minimized (10).

Adequate and correct annealing of templates and mutagenic primers is crucial for the synthesis of mutant DNAs. In the thermolabile polymerase-based non-PCR, the DNA template and the primer are encouraged to anneal with each other through a continuous or stepwise decrease in incubation temperature to below the estimated melting temperature, which is often room temperature. Additional cooling of the DNA template and primer complex on ice improves subsequent DNA synthesis (8, 10). When DNA templates were initially denatured by boiling, the snap-cooling of denatured templates on ice was shown to produce a better yield of mutant DNAs than the stepwise cooling to 25°C and then to 4°C (11).

When relatively long primers (for example, as required by deletion or insertion mutagenesis) are used for DNA synthesis with a thermolabile enzyme at 37°C, these primers may bind to incorrect regions of the template, and thus not produce the DNA products intended. To avoid such mis-priming, Hofer and Kuhlein (12) added Klenow at 65°C to the annealed template and primer complex and allowed the DNA synthesis reaction to proceed for 5 min at this higher temperature before cooling it to 37°C and adding more Klenow. The correct mutant DNA was obtained by this unusual treatment. Presumably at 65°C, residual activity of Klenow was present, which correctly extended and locked the primer onto the template. DNA synthesis then continued successfully at 37°C.

Secondary Structures and GC-Rich Regions

In the thermolabile polymerase-based non-PCR, alkali and/or heat denaturation are used to separate two strands in order to allow binding of the primer and to destroy the local conformation of each strand, so that the DNA synthesis can proceed. DNA regions that are rich in guanine (G) and cytosine (C) may hinder the separation of the two strands, whereas secondary structures may slow down or stop DNA synthesis. This type of structure is particularly abundant in mammalian and viral DNAs, and may even exist in DNA vectors, e.g., pBluescript. In one study, no mutant DNAs were produced until the same insert was switched from a pBluescript into a pUC18 vector (4). Such complex structural conformations thus can be an impediment to efficient DNA mutagenesis using thermolabile polymerases.

Among thermolabile enzymes, T7 DNA polymerase is more capable of overcoming secondary structures than T4 DNA polymerase (Bio-Rad Technical Bulletin 1625; 5). Sequenase version 2, derived from T7 DNA polymerase, produces two to five times as much mutant DNA as T4 DNA polymerase does in parallel experiments (5). Klenow, however, with its strand-displace-

162   LING AND ROBINSON

TABLE 2

Comparison of Two Types of *in Vitro* DNA Synthesis Used in Mutagenesis

	PCR	Non-PCR
Reaction temperature/duration	Cyclic, typically 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), extension (72°C, 1 min)	Constant at 37°C for 1 h
Strands synthesized	Two strands	Only one strand
DNA polymerase	Thermostable, such as Taq and Pfu	Thermolabile, such as Klenow, T4 DNA polymerase
Primers required	At least two, sometimes more, one of the two may be mutagenic	Usually just one as the mutagenic primer, sometimes an extra one as the selection primer
Fold of amplification	10^2–10^8	<1
Product synthesized	Shorter, linear double-stranded DNAs, usually containing only the coding region of a gene	Longer, hybrid plasmid, phage or phagemid DNAs carrying linear mutant strand and circular wild-type strand
Sequence fidelity	Relatively low	Relatively high
Hindrance due to secondary structure GC-rich region	Not likely	Likely
Cloning	Inserted into a vector by ligation	Circularized by ligation

ment activity, is more capable of overcoming certain secondary structure problems than T7 DNA polymerases or sequenases (6). Additives such as *E. coli* single-stranded DNA-binding protein can be employed to overcome secondary structure problems (7). DMSO (8) and certain weak base-pairing deoxyguanosine or deoxycytosine analogs, used in DNA sequencing to overcome the so-called compression problem caused by secondary structures, may also be used to alleviate secondary structures in DNA mutagenesis (9).

In summary of the basic procedures of DNA synthesis or DNA mutagenesis, a comparison of various aspects of the thermostable polymerase-based PCR and the thermolabile polymerase-based non-PCR are listed in Table 2. Novice readers are advised to consult additional references (86, 13). In addition to the *in vitro* DNA synthesis (PCR and non-PCR), the *in vivo* DNA mutagenesis methods will also be described briefly in the subsequent section. Basically, the various methods or protocols for *in vitro* (PCR or non-PCR) and *in vivo* DNA mutagenesis are assembled by manipulating the DNA templates, the primers, the synthesis materials (dNTP), or the synthesis conditions to meet different kinds of mutagenesis needs, such as SDM, REM, deletion and nested deletion, insertion, and scanning mutations. To generate a mutant DNA, in some cases both *in vivo* and *in vitro* methods, or both thermostable polymerase-based PCR and the thermolabile polymerase-based non-PCR methods, are used in the protocol, thus making the classification less clear.

APPLICATIONS OF PCR IN DNA MUTAGENESIS: MUTANT-DNA CONSTRUCTION STRATEGIES

Site-Directed Mutagenesis

In SDM, a specific mutagenic primer is used, resulting in a specific mutant with a predetermined site

and type of mutation. Although an amino acid can be replaced by any of the remaining 21 amino acids, possibly even by some artificial amino acids (101), some substitution mutations may result in a drastic global conformational change of the protein or a mistargeting to its site of action. Such a change will almost certainly bring about a significant change in function. Therefore, for studies aiming to map out functionally important amino acid residues in a protein, changes in the residues that alter only the local conformation of a protein are generally desirable. Guidelines for preferred and avoided substitutions have been suggested for this purpose (102).

The following described methods or protocols are generally used for SDM and are classified according to the desired position of the mutations in the PCR product.

Introducing Mutations into the Terminus of a PCR Product

Mutations are simply introduced by one of the two primers, and thus mutations are located within approximately 30 bp of either end of a PCR product. A pair of restriction sites is also usually built into these two primers. The resulting PCR product can then be readily cloned into a vector with one or two compatible restriction sites (62).

Introducing Mutations into the Middle of a DNA (Connecting PCR)

In vitro ligation of two PCR products carrying mutations. To create mutations in the middle of a relatively large DNA, two separate PCRs can be performed to amplify two halves of a complete gene, using four primers (Fig. 1A). An outside-forward primer is paired

APPROACHES TO DNA MUTAGENESIS: AN OVERVIEW   **163**
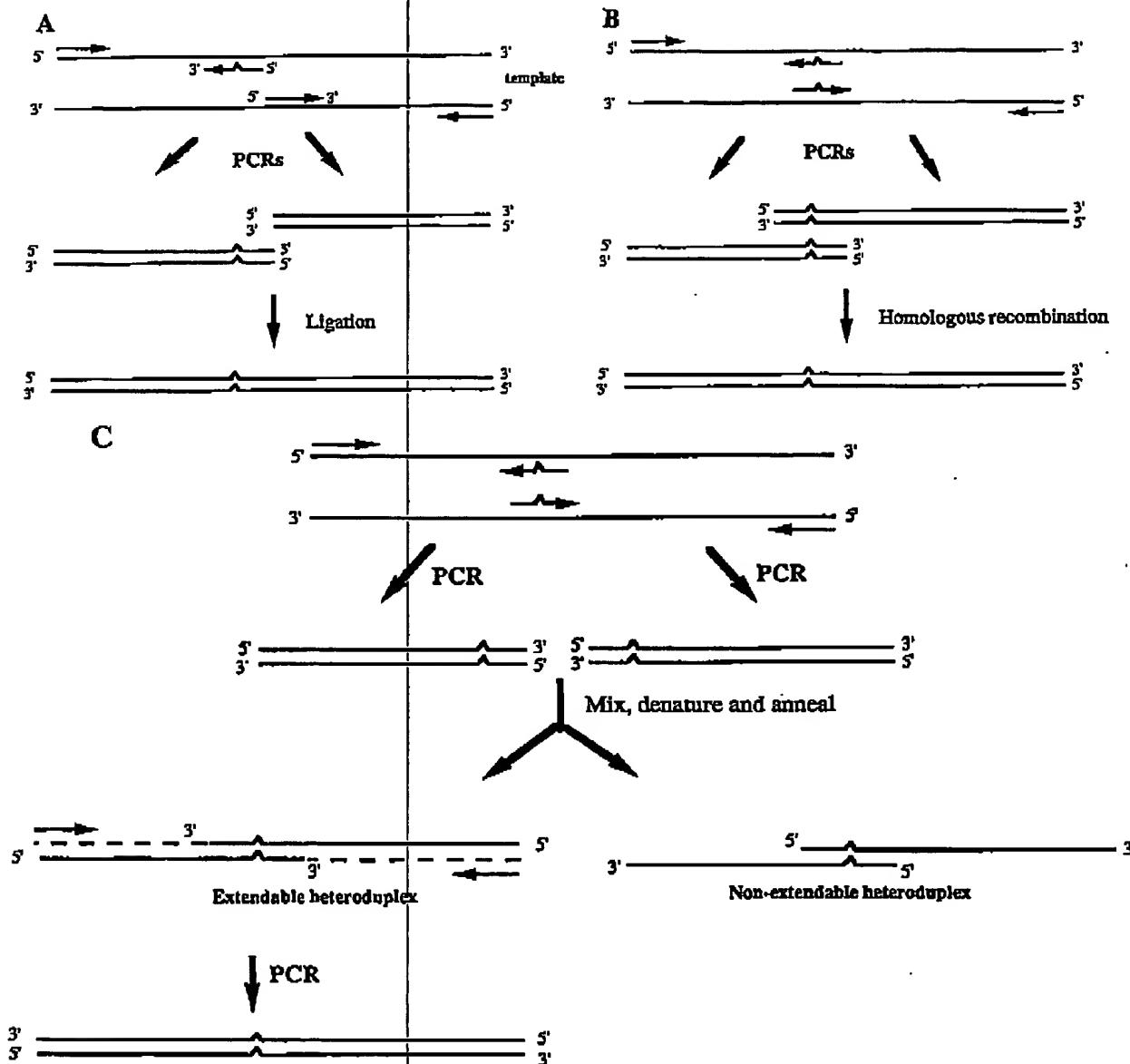
FIG. 1. Introducing mutations into the middle of a DNA (connection PCRs). (A) Introducing mutations by *in vitro* ligation of two PCR products. Two separate PCRs are performed to obtain two halves of a completed gene. The resulting two PCR products are then ligated to form a fused DNA carrying mutations situated in the middle and introduced by middle primers. (B) Introducing mutations by *in vivo* homologous recombination. Two PCRs are performed to generate two fragments carrying overlapping sequences introduced by two middle primers. The outside ends of these two fragments are ligated into a vector and transformed into the bacteria. As a result of the homologous recombination *in vivo* in the bacteria, one of the two overlapping sequences is removed and two fragments are joined together. (C) Introducing mutations by *in vitro* overlap-extension PCR. Two PCRs are performed to produce two fragments that carry overlapping sequences. These two fragments are then mixed, denatured, and annealed in a PCR-ready buffer to obtain mutant DNAs in a further PCR.

with a middle-reverse primer to synthesize the first half of the gene; an outside-reverse primer is paired with a middle-forward primer to synthesize the second half. These two half-fragments which do not overlap are thus aligned in "tail-to-tail" fashion. The two resulting PCR products can be phosphorylated and then

164   LING AND ROBINSON

ligated to form a fused DNA carrying mutations situated in the middle (60, 63–65). One or both of the middle primers can be mutagenic. This fused mutant DNA can be subcloned directly or can be amplified further using two outside primers before subcloning (66, 67).

Alternatively, a common restriction site can be built in at the 5'-terminus of both middle primers. The resulting two PCR products can be digested and then ligated. Sometimes, the incorporation of a restriction site into the middle primers causes an undesirable alteration to the corresponding amino acid sequence of a protein. To circumvent this problem, a sequence for a unique Class IIs restriction enzyme can instead be tagged onto the 5'-terminus of both middle primers (68, 69). These tag sequences are completely removed by digestion with a Class IIs enzyme.

Usually, ligation is carried out with thermolabile T4 DNA ligase. A thermostable *Taq* DNA ligase has also been used along with *Taq* DNA polymerase in a ligase chain reaction (LCR) (65). After two primary PCRs, the resulting two fragments are mixed with wild-type templates to undergo LCR, by which two fragments are connected. In a similar method (70), a phosphorylated mutagenic primer and two outside primers were used to perform PCR and LCR concurrently. A 900-bp mutant DNA was generated. Although the use of *Taq* DNA ligase simplifies the procedure, its low yield of final ligated product makes it less desirable. The desired full-length product is also often contaminated or mixed with partial-length by-products.

In vivo homologous recombination of two PCR products carrying mutations. Homologous recombination *in vivo* in bacteria is a DNA-joining event between two DNA ends carrying the same or very similar sequences (mutually overlapping sequences; Fig. 1B). As a consequence of this homologous recombination, one of the two overlapping sequences is removed; the two fragments are joined together in the bacteria. Two PCRs are performed to generate the two initial fragments carrying overlapping sequences introduced by two middle primers. Mutations are also introduced by two middle primers. To ensure the presence of mutation sequences in all or most recombinants, mismatches are best placed in both overlapping sequences, or in the portion of a middle primer that does not overlap with the other middle primer (23).

After two PCRs, both outside ends of these two fragments are ligated into a vector. This linear DNA is then transformed into bacteria to restore a circular functional plasmid via *in vivo* homologous recombination (23). Mutagenesis efficiencies around 50% may be obtained.

In vitro overlap-extension PCR. Overlap-extension PCR was developed almost concurrently by Higuchi and associates (70) and by Ho and colleagues (71). (Interest-

ingly, most subsequent papers only cited Ho *et al.*). Two PCRs are performed to produce two DNA fragments that carry overlapping sequences (Fig. 1C) and intended mutations, both of which are introduced by two mutagenic middle primers. Two PCR fragments are then mixed, denatured, and annealed in a PCR-ready buffer, to generate two heteroduplexes via overlapping sequences. Only one of the two heteroduplexes, which carries two 3'-termini at the joint, can be extended by *Taq* DNA polymerase to form a full-length double-stranded mutant DNA. The other heteroduplex, since it carries two 5'-termini at the joint, is not extendible. The extended double-stranded mutant DNA is amplified in a further PCR using two outside primers.

The limitation of this overlap-extension method is that when the intended mutant DNA is longer than 600 bp, or when the intended position of mutations is located near either end, the yield of desired mutant DNAs drops dramatically (72). Nonetheless, when an additional extension step and long PCR technology were used (31), it was possible to prepare mutant DNAs as large as 3.1 kb, even when GC content was over 60%.

Introducing Mutations Anywhere in a Final PCR Product

The megaprimer PCR method. Conventionally, primers or oligonucleotides are chemically synthesized, single-stranded, and relatively short. Then, it was observed that double-stranded DNAs of several hundred base pairs can also serve as primers for DNA synthesis or mutagenesis (5, 74). Because these primers are larger than conventional ones, they are called megaprimers.

In the megaprimer mutagenesis method, two outside primers, one middle mutagenic primer (reverse or forward, whichever direction makes the megaprimer smaller), and wild-type templates are needed (Fig. 2). The first PCR, using one outside primer, the middle mutagenic primer, and wild-type templates, is performed to form a double-stranded megaprimer containing mutations introduced by the mutagenic primer. In the earlier method (Fig. 2), this megaprimer is then purified and used together with the other outside primer and wild-type templates to obtain the final mutant product in a second PCR (35, 75). In a more recent modified method (Fig. 2), the purified or nonpurified megaprimer is extended by using wild-type templates (17). Only one strand of this double-stranded megaprimer is extendible to form a single-stranded full-length mutant template, which is then amplified by two outside primers in a second PCR to generate a large amount of double-stranded mutant DNA (35, 74, 76).

The template-independent addition of a nucleotide (most frequently adenosine) to the 3'-terminus of a product by *Taq* DNA polymerase is often seen in PCR

APPROACHES TO DNA MUTAGENESIS: AN OVERVIEW   165
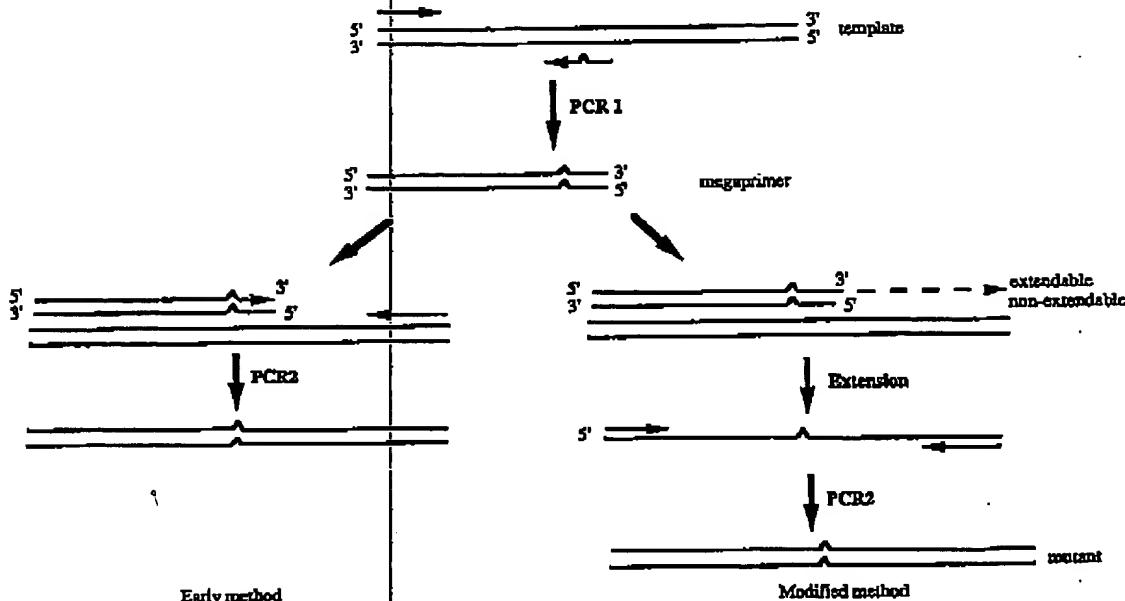
FIG. 2. The megaprimer method. A PCR is performed to form double-stranded megaprimer. Early method: This megaprimer is then purified and paired with an outside primer to obtain the final mutant product in a second PCR. Modified method: The megaprimer formed in the first PCR then is extended to form a single-stranded mutant template which is used to further amplify the final mutant product in a second PCR.

products. More often than not, this terminal addition is a mismatch for the megaprimer in relation to its template, thus stalling the extension of the mega-primer. Sometimes such 3'-terminal mismatches are tolerated (77), but the result is an unintended change to the amino acid sequence. Sharrocks and Shaw (78) suggested that the 5'-terminus of the mutagenic primer should be preceded immediately by a wobble base of a codon. Thus, in many cases, the addition of a mismatched base at the 3'-terminus of the megaprimer does not alter the amino acid sequence. Another interesting solution to this problem is to design the mutagenic primer in such a way that its 5'-end is immediately preceded by a T (79). In this way, any A added by the 3'-terminal transferase activity of Taq DNA polymerase does not interfere with the subsequent extension of megaprimers or change in amino acid sequence..

Alternatively, a universally applicable strategy is to attach a Class IIs enzyme-recognition sequence to the 5'-terminus of the mutagenic primer (80). Before the extension and the second PCR, the megaprimer formed from this mutagenic primer can be purified and digested with this enzyme, which removes residues of recognition sequences along with any 3'-terminal mismatches. Finally, both Pfu and Vent DNA polymerases, which lack 3'-terminal transferase activity, are superior to Taq for use in the megaprimer PCR method (17, 18).

Megaprimers, being long, double-stranded DNAs, are often difficult to denature, to anneal, and to extend to form full-length mutant templates. The megaprimer can be extended more efficiently by lengthening the times for the denaturation, extension, and (particularly) annealing in the extension step (19, 54, 81, 82), or by allowing additional cycles for the extension step (83). Several strategies are also used to selectively enrich the useful strand of the megaprimer, e.g., by performing an asymmetric PCR (19, 73) or by separating it from the other strand by biotin-labeling the corresponding outside primer used in the initial PCR (84).

Improved megaprimer methods. Picard and co-workers (18) attempted to combine the three stages of the megaprimer method. For the first 10 cycles of PCR, small amounts of both the mutagenic primer and one outside primer were added in a reaction tube to form the megaprimer. For cycles 11–20, a relatively large amount of the other outside primer was added, allowing the amplification of full-length mutant DNAs. For cycles 21–30, amplification was further encouraged by adding more of both the outside primers. Several interventions were required for this one-tube PCR, and product yields were low.

The megaprimer method recently has been improved further by the authors, so that it is now practical to form a large megaprimer with a high yield of final products (19). This makes it possible to mutagenize a large gene cassette without any unique restriction enzyme

166   LING AND ROBINSON

sites in the region of interest. It is now also more conve-
nient than before: the operator can now set up a three-
stage PCR in one tube (much like a routine PCR) and
allow programming software to run it automatically
without any human intervention. All required compo-
nents, including three primers (two outside and one
mutagenic), are placed together with DNA templates
and a DNA polymerase in one tube at the beginning of
the PCR. The basis of this method is that each stage
of this PCR is optimized specifically for a single func-
tion by manipulating conditions, such as the number
of cycles, the concentrations of mutagenic primers and
templates, and the use of a partial dideoxyNTP 3'-end–
blocked template. The product from the first stage is a
megaprimer containing the mutation; from the second
stage, a full-length mutant template; and from the final
stage, a mutant DNA in sufficient amounts. This modi-
fied One-STEP (One-Step Three-stage Efficient PCR)
method not only has made the megaprimer method
more convenient, but also has increased the sizes of
the megaprimer to at least 1.3 kb and the final mutant
DNA to at least 5.4 kb, with high yields.

   The Inverse PCR method. Inverse PCR is commonly
used for cloning unknown genomic sequences (e.g., 5'-
or 3'-untranslated regions or introns) and has subse-
quently been used for sequencing unknown cDNA (89)
(Ling and Robinson, manuscript in preparation). The
inverse PCR method can also be used in mutagenesis
(90), in which a pair of tail-to-tail primers is made from
the site of the desired mutation and used to amplify the
entire double-stranded plasmid containing the desired
gene (Fig. 3). To introduce mutations, one of the two
primers used is mutagenic. The resulting linear double-
stranded PCR product is then circularized by ligating
the two blunt-ends. To allow such a ligation, phosphate
must be added to the 5'-terminus of the PCR products,
by phosphorylating either the primers in advance or
the PCR products afterward. The ligated DNA is then
transformed into *E. coli*.

   A unique restriction site may also be incorporated
into both primers to create compatible cohesive ends
in the PCR product, thus increasing the efficiency of
circularization and removing the requirement for phos-
phorylation. In this case, if possible, either degenerate
codons for amino acids that do not change the peptide
sequence or a Class IIs enzyme sequence should be
used (58). Alternatively, overlapping sequences can be
generated at two ends of this linear double-stranded
DNA through the two primers. The *in vivo* intramolecu-
lar homologous recombination in bacteria will circular-
ize this product (91, 92). Instead of directly trans-
forming this PCR product into bacteria, Jones and
Winistorfer (93) have found that denaturation and re-
annealing prior to transformation seemed to consis-
tently yield 2- to 11-fold more transformants than di-
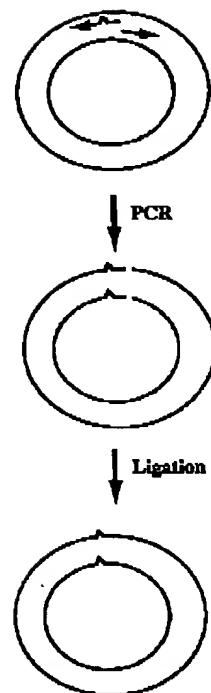
FIG. 3. The inverse PCR method. A pair of tail-to-tail primers is
made from the intended mutation site and used in a PCR to amplify
the entire plasmid containing the desired gene. This linear double-
strand plasmid is then circularized and transformed into *E. coli*.

rect transformation. Likely, this treatment renders the
two ends cross-annealed among the four strands, thus
facilitating recombination *in vivo*.

   Since it is often difficult to gel-resolve and purify the
desired linear mutant PCR product from the circular
wild-type plasmid template, the mixture of both is gen-
erally used to transform *E. coli*, resulting in a lower
mutagenesis efficiency (92). The wild-type template
should therefore be used in a low concentration (93–
95). For example, the use of 100 ng plasmid DNA in
the original method by Jones and Howard (91) resulted
in a relatively low mutagenesis efficiency: less than
20%. However, when 10 pg to 1 ng DNA was used, more
than 50% of transformants were mutant colonies (92).
Another way to minimize wild-type background after
transformation is to use alkali-denatured plasmid DNA
templates (95, 96), which is beneficial probably because
the alkaline treatment not only denatures the template
more efficiently, but also makes the template less effi-
cient in bacterial transformation. Coincidentally, Dor-
rell *et al.* (95) found that mutagenesis efficiency
increased significantly when this pretreatment of tem-
plates was combined with the omission of phenotype
recovery after the heat-shock step during *E. coli* trans-

formation. It is thus postulated that damage to wild-type templates caused by alkaline treatment is not repaired as a result of omitting the phenotype recovery step. Since newly synthesized mutant DNAs are not treated by alkali, they are thus not damaged.

This inverse PCR method is very simple and effective, and has been utilized to generate mutant plasmids as large as 11.5 kb (24), although it generally works best for plasmids under 3.1 kb.

Random and Extensive Mutagenesis

The REM approach is extremely useful when many mutations at a given position or many mutations at different positions are needed. For example, the REM approach has been used successfully in mapping out the important residues of proteins such as *Taq* DNA polymerase I (103) and *Eco*RV restriction endonuclease (104). The REM approach has also been used to engineer antibody specificity (60, 105) and enzyme specificity, such as triose-phosphate isomerase (106) and aspartase (107).

Using Primers Randomly Spawned with Mismatched Bases or Ambiguous Bases

In the REM, a set of mutagenic primers is generated in one synthesis reaction by supplying three mismatched bases at a single desired base position. This set of primers is then used to synthesize a set of mutant DNAs with three different mutations at the same position. Multiple sets of mutagenic primers can also be synthesized at once (one synthesis reaction) by providing a high concentration of the correct base and low concentrations of the three wrong bases at the multiple desired REM sites. Instead of supplying three mismatched bases for primer synthesis, deoxyinosine phosphoramidite (dI, an ambiguous base) may be added to a dNTP phosphoramidite reservoir at one or several base positions. The dI-containing primer does not specify the complementary base in the subsequent DNA synthesis. Since all four natural bases may pair with deoxyinosine, there is a 75% probability that any one of the three wrong bases, instead of a correct base, is paired with this ambiguous base. In some cases, trinucleotide phosphoramidite units representing codons for all 20 amino acids can be used to synthesize oligonucleotides. Those random mutagenic oligonucleotides with all possible amino acid mutations can be obtained quickly (108), although such oligonucleotide synthesis is too specialized to be useful to most investigators.

Using Erroneous PCRs

DNA synthesis *in vitro* tends to produce random sequence errors. This tendency is relatively great in the thermostable-polymerase PCR method, particularly

when *Taq* DNA polymerase is used. *Vent* and *Pfu* DNA polymerases make far fewer errors than does *Taq*. The error rate found by Zhou and colleagues (109)—that 35% of all subcloned PCR products synthesized by *Taq* contained at least one base substitution in a 633-bp product—is somewhat higher than that reported by Lerner and associates (110), who used a similar method.

Measures can be taken to increase the error rate of PCR for REM (1, 109, 111, 112). Factors that can be used to increase the error rate of a PCR include buffer composition (e.g., a high magnesium concentration, a high pH, or the addition of 0.5 mM MnCl₂) and other conditions (e.g., a large amount of polymerase, a small amount of template, a great number of cycles, a long extension time, or a low annealing temperature). In addition, a biased pool of the four dNTPs, with concentrations differing by a factor of 10 to 1000, also encourages a higher error rate (111–114). Finally, the template may be treated chemically to encourage misincorporation in the DNA synthesis. Although the treatment itself alone is inefficient at inducing random mutations, it is effective when combined with an error-prone PCR (115, 116).

Under various combinations of these conditions, an error rate of 1 bp per 150 bp can be obtained per PCR. Thus, on average, every clone containing a gene of 150 bp is expected to have a single random mutation.

On the other hand, undesirable multiple mutations can occur when a highly error-prone PCR is used, which are caused by the compounding of mutations at various cycles. One possible solution to this problem is to amplify DNAs first in an error-minimized PCR for many cycles, and then in an error-prone PCR for only limited cycles.

A drawback of the PCR-based REM method is that different genes or different regions of the same gene tend to have different error rates, even when the same conditions are applied (unpublished observations from our laboratory, and 117). The resulting mutants also tend to have more transitions (A to G, or T to C) than transversions (A to C, T to G, A to T, or G to C).

Using Deoxyinosine or Other Degenerate Base Analogs

Deoxyinosine triphosphate or other ambiguous degenerate base analogs may be incorporated into PCR products when the analog is added to a mixture of three dNTPs at normal concentrations and the fourth dNTP at a lower concentration. This biased set of dNTPs forces dI to be incorporated in place of the fourth base during DNA synthesis. This dI-containing DNA then serves as a template for subsequent cycles of DNA amplification. Theoretically, there is a 75% probability that wrong bases may be incorporated wherever a dI

is located, since this dI complements weakly with all four natural bases (42). Spee and co-workers (118) found that using this method, up to 4 bp mutations per 1000 bp could be produced per PCR, which on average corresponds to a 100% mutagenesis efficiency for each clone containing a 250-bp PCR product. Under a different PCR setting, using 0.2 mM dITP in addition to 0.2 mM each dATP, dCTP, dGTP, and dTTP, Ikeda and colleagues (105) obtained 19 mutants carrying a single mutation out of 52 screened clones for a 300-bp PCR product.

PCR methods using dI or other degenerate base analogs seem to be less sequence-dependent than error-prone PCR methods do, and also seem to have less bias toward transitions against transversions (1).

Scanning Mutations

Scanning mutagenesis or linker scanning mutagenesis is a technique designed to systematically change sequence segments in a regular increment (e.g., every 20 bp) into a common sequence (e.g., 2–5 bp) within a gene. Thus, a set of scanning mutant DNAs can be obtained to map out functionally important regions of a protein quickly.

An example is the so-called alanine scanning mutagenesis, in which amino acids at different positions of a protein are replaced systematically with alanine. The reason for choosing alanine as the common replacement amino acid is that it does not change the peptide-chain orientation, as do glycine and proline, and also it does not possess extreme steric and electrostatic characteristics (123). Cysteine can be used as a common residue as well to probe whether or not a region of a protein is membrane-spanning, since cysteine interacts with a readily detectable membrane-permeable thiol reagent. N-ethylmaleimide (124). A protease-cleavage site can also be incorporated as a common sequence, to identify quickly the corresponding mutant protein by protease digestion (125). In scanning mutagenesis studies of DNA structure and function correlation (e.g., promoter DNA), the common sequence usually contains a restriction site to facilitate the screening of mutant DNAs (126).

Overlap-extension or megaprimer PCR methods may be used to create such scanning mutants (127, 128).

Deletions and Nested Deletions

Small deletions (less than 10 bp) can be made easily by an oligonucleotide primer design. For larger deletions, relatively long primers can be used, which loop out the sequences to be deleted. A 152-bp intron has been deleted successfully in this way by using a 30-bp primer (129). Larger deletions may also be produced by joining two PCR-amplified fragments, leaving out a portion of a DNA fragment (30).

Nested deletion of DNAs is another way to map out functionally important residues of a protein (130). Traditionally, a linear DNA can be deleted stepwise from one end by a exonuclease III digestion followed by a S1 nuclease digestion. Both the amount and the duration of enzyme digestions can be adjusted carefully to produce a set of nested-deletion DNA mutants. DNAs can also be labeled randomly with thio-dNTPs before the exonuclease III treatment. Since thio-containing sequences are resistant to exonuclease digestion, wherever the thio-dNTP is incorporated into the DNA, the digestion will stop. In this way, digestion can be controlled readily to obtain different incremental fragments (131). A more convenient and increasingly popular method is to amplify a set of nested deletions with a common primer from one end by PCRs, paired with a series of primers made by varying positions from the other end. Obviously, numerous oligonucleotides are required.

Insertion

Small insertions (fewer than 10 bp) can be made easily by designing mutations into primers. Relatively long inserts should be attached to the 5'-terminus of a primer when used in an inverse PCR method. Long insertions can be made by the use of the megaprimer or an the overlap-extension PCR. An insertion sequence can also be placed in the middle of a relatively large primer for mutant strand synthesis (132). For insertions greater than 200 bp, three individual fragments, one of which is the inserting fragment, can be produced by PCRs and subsequently joined by ligation (30, 133).

Multiple Mutations

Multiple mutations are sometimes required in the same gene for studies of whether a second mutation site modulates another (134). Multiple mutations can be obtained simply when several mutagenic primers are used one at a time for several rounds of mutagenesis (135). Sometimes, desired multiple mutations can be obtained even when all mutagenic primers are placed at the same time in the same reaction (136). Alternatively, a few DNA fragments, each carrying mutations, can be connected to generate a joined product with multiple mutations (65).

APPLICATIONS OF PCR IN DNA MUTAGENESIS: MEASURES OF MUTANT SELECTION

Nested PCR

For the megaprimer PCR method, the challenge is to amplify selectively the mutant template in the second PCR, without concurrent amplification of the coexisting wild-type template. A popular nested PCR strategy is

to use a tagged outside primer in the first PCR. This tagged sequence, 10–30 bp long, is located at the 5'-end of the primer and is absent in the wild-type template (Fig. 4A). The resulting tagged megaprimer is then extended to form a single-stranded tagged mutant template by using wild-type templates. Then a new primer (often called the nested primer), made mostly from this tagged sequence, is used in the second PCR. Since this nested primer recognizes the tagged mutant template, not the wild-type template, primarily mutant DNAs are amplified. This strategy thus has increased significantly the efficiency of the megaprimer method (65, 149).

Instead of a nested primer, a template different in sequence only at one end from the first wild-type template is used for the extension of the purified mega-primer to form a single-stranded mutant template (Fig. 4B). This pair of templates can be obtained by cloning the insert into two different vectors. Since one of the two outside primers used in the second PCR will not bind to this template with a different end, only the mutant templates will be amplified (150, 151). A similar approach is to use two templates, differing in the orientation of the insert relative to flanking vector sequences by cloning an insert into the same vector in two opposite orientations, or into two related vectors differing in only the orientations of cloning sites, such as pUC18 and pUC19. Thus, when one template is used for the first PCR and the other for megaprimer extension, without the purification (Fig. 4C) the resulting mutant template contains the same sequence on both sides and is therefore amplified in the second PCR by using only one outside primer derived from that sequence. In other words, this one primer functions as both a forward and a reverse primer; neither wild-type templates (orientation 1 or 2) will be amplified with this primer alone (152).

Steinberg and Gorman (82) and Liang and colleagues (99) capitalized on the use of two different templates, simply by using a piece of short DNA as a template for megaprimer extension (Fig. 4D). This short DNA, made by restriction digestion of the wild-type template or by PCR, supports the extension of the purified megaprimer but does not support the amplification of the full-length wild-type template in the second PCR (82, 99). To avoid the extension of this short DNA itself to form a full-length wild-type template, the 3'-end of this short DNA is blocked by adding dideoxynucleotides. Alternatively, this short template is made to carry an end nonhomologous to the megaprimer. In addition to using a short fragment for megaprimer extension, the template for the first PCR can also be a shorter fragment, lacking the opposite end of the megaprimer (153).

Purification of Mutant Products

Other than the above-described nested PCR, probably the most straightforward selection procedure for

PCR products is the purification of mutant DNAs from wild-type template DNAs by biotinylating one of the two primers for PCR (4). Thus, the newly synthesized mutant strand can be purified from wild-type templates. This biotinylated mutant strand alone can then be used as a template for the synthesis of the second strand. The resulting double-stranded mutant DNA can be circularized by digesting off the biotin cap with an enzyme, the site of which is built into the biotinylated primer (4). Finally, whenever the template and mutants are different in size, mutant PCR products may be purified from templates by simple gel electrophoresis.

APPLICATIONS OF PCR IN DNA MUTAGENESIS: SUBCLONING OF MUTANT PCR PRODUCTS

Most often, the mutant insert generated by PCR needs to be subcloned into an expression vector for protein studies. A recent review summarized several common ways to clone PCR products, including the purification of PCR products, the incorporation of restriction sites, the 3'-T-protruding vectors for cloning 3'-A-protruding products, and the cloning of blunt-ended products. Several aspects other than those discussed in Schaefer (157) are summarized below.

Recent Improvements on Ligation

Under the standard PCR conditions, the 3'-ends of some PCR products often are not extended completely; usually, they are missing the last several base pairs where restriction sites are designed through primers. Thus, PCR products often cannot be digested, leading to low efficiency or even failure of a ligation. An additional treatment of PCR products with *Pfu* DNA polymerase or T4 DNA polymerase combined with dNTP has been used to fill in the two product ends completely. Extra extension time (5–10 min, or even 60 min) at the end of a PCR process also helps to increase the efficiency of cloning (158).

Sometimes, when restriction sites are built too close to the end of a PCR product, those sites will digest with difficulty, even when the ends of those products are complete. Another alternative to increase the ligation efficiency is to create sticky ends in PCR products by partial digestion with exonuclease III.

It is also possible to create a sticky end without any digestion. The desired PCR product is amplified separately with two pairs of primers that differ only in that each primer in one pair is longer by 3 bp (e.g., GGG) at its 5'-end than that of the other pair. These two products are mixed in about equal molar ratio, denatured, and reannealed. Among all reannealed molecules, 25% will have 5'-GGG-protruding ends, and 25% will be 3'-CCC-protruding. Each molecule with sticky ends is then cloned easily into vectors

170   LING AND ROBINSON

A

template

PCR1

megaprimer

template

Extension

nested primer

PCR2

B

template

PCR1

purified megaprimer

template with
a different end

Extension

PCR2

C

orientation-1
template

PCR1

orientation-2
template

Extension

PCR2

D

full-length template

PCR1

purified megaprimer

short template containing
a non-homologous end

OR

5' ——————— 3' short template containing
3' ——————— 5' a blocked end

Extension

PCR2

FIG. 4. Nested PCRs. (A) Nested primer. After the mutant template is formed, a nested primer (shown boxed and with an arrowhead) is used to selectively amplify the mutant template in the second PCR. (B) A different wild-type template. After the megaprimer is produced, it is extended on a template with a different end from the megaprimer, rather than the original template, to form the mutant template. The mutant template is then amplified selectively in the second PCR. (C) Two templates with different insert orientations. The megaprimer is produced by using the orientation-1 template in the first PCR. It is then extended on the orientation-2 template. This mutant template is amplified using only one outside primer. (D) Short template. After the megaprimer is amplified, it is extended on a short template. The mutant template is then amplified in the second PCR.

carrying compatible synthetic linkers. This nondiges-
tion method gave significantly better cloning effi-
ciency than TA cloning (159).

Ligation of vectors and inserts, both carrying blunt
ends, is often difficult (160, 161). PCR thus may be
used to amplify a product from the ligated molecule,
carrying restriction sites that are easy to digest. Trans-
formation of a secondary ligation would produce more
of the desired clones (160, 161).

Usually, PCR products are purified from *Taq* poly-
merase by phenol and chloroform extraction, and from
primers, dNTPs, and buffer components by gel electro-
phoresis and ethanol precipitation. However, even
after these treatments, residual DNA polymerases and
dNTPs may still be present in these PCR products,
which modify restriction ends after the digestion. For
example, 3'-recessive ends can be filled by *Taq* poly-
merase, and 3'-protruding ends can be removed by
Vent or *Pfu*. Such alterations to the digested ends pre-
vent subsequent ligation. Therefore, thorough extrac-
tion (three times) with phenol and chloroform is recom-
mended by Benett and Molenaar (162). In some cases,
even repeated phenol–chloroform extraction is not suf-
ficient to remove *Taq* polymerase, which then must be
degraded by a proteinase K digestion (163).

Primers, by default after their chemical synthesis,
and hence PCR products, are usually not phosphory-
lated at their 5'-ends. Thus, vectors for cloning must
be phosphorylated to allow the ligation of at least one
strand between the vector and the PCR product. When
both vectors and PCR products are phosphorylated, the
efficiency of ligation increases greatly (164). Cyclic
changes in incubation temperature between 10 and
30°C every minute also increase the ligation efficiency
by 4- to 8-fold, compared to the constant temperature
incubation (165). The transformation efficiency can
also be increased greatly when the ligase is heat-dena-
tured and products of the ligation reaction are purified
from the buffer (166).

Ligation-Free Methods

PCR products may be inserted into a vector *in vivo*
via homologous recombination in *E. coli* (167–169).
Two primers used for the PCR are each attached with
an overlapping sequence derived from the vector. As a
result, each end of this PCR product has an overlapping
sequence, which is homologous to its vector end. The
vector and the insert are then transformed into a bacte-
rial host and connected directionally via intermolecular
homologous recombination. The minimal length of this
kind of overlap sequence seems to be around 23 bp
(168, 169). *In vitro* predenaturation and reannealing
prior to transformation have been shown to increase
this cloning efficiency (93). Long sticky ends (usually
12 bp or greater) compatible between a vector and an

insert can also be created by choosing or designing re-
striction enzyme ends in the vector which are made of
only three nucleotide monophosphates in their termi-
nal 12 bp or longer sequences. Treatment with T4 DNA
polymerase in the presence of the fourth dNTP, and
the absence of the three other dNTPs, will then remove
nucleotides from one strand of the chosen terminal se-
quence, creating sticky ends. Both the insert and the
vector are treated separately, creating terminal se-
quences that overlap. This cloning-free method is very
efficient. Almost 100% of transformants have been
found to be desirable clones (170).

Finally, primers can be designed to be rich in dUMP.
The resulting PCR product, which contains dUMP at
its ends, can then be digested with UDG, leaving 3'-
protruding ends of about 12–15 bp. This fragment can
be cotransformed into *E. coli* with vectors, carrying
compatible 3'-protrusions made by using synthetic
linkers. The yield of transformants of this method is
high (171, 172), although dUMP-containing primers
sometimes failed to amplify PCR products when *Pfu*
and *Vent* were used (173).

APPLICATIONS OF *IN VITRO* NON-PCR AND *IN VIVO* METHODS IN DNA MUTAGENESIS

Mutant-DNA Construction Strategies

Assembling Double-Stranded Oligonucleotides Carrying Mutations

The simplest strategy for mutant DNA constructs
of small size is to assemble a double-stranded DNA
with a pair of cross-annealed oligonucleotides car-
rying desired mutations. This constructed DNA can
be used to replace the wild-type counterpart by using
naturally existing or artificially created restriction
sites (55, 56). It is important that these sites be cre-
ated in such a way that their creations do not unin-
tentionally affect the sequence (57). Because most
amino acid residues in a protein are encoded by mul-
tiple codons, it is possible to alter the DNA sequence
to accommodate a restriction site without changing
the amino acid sequence (58).

Several phosphorylated, mutually complementary
oligonucleotides can be used to assemble a long dou-
ble-stranded DNA (2, 59). Their neighboring ends can
be ligated directly. If singled-stranded gaps remain
after ligation, they can be filled in by a thermolabile
DNA polymerase or alternatively by PCR, and then
ligated with each other (60). Mutations are built in by
swapping one of the numerous oligonucleotides with
a mutagenic one (61).

Although simple, this procedure is limited to rela-
tively short DNAs, generally 200–300 bp, otherwise
too many oligonucleotides are required, making the
procedure expensive. This strategy can be used to the
applications of SDM and REM.

172   LING AND ROBINSON
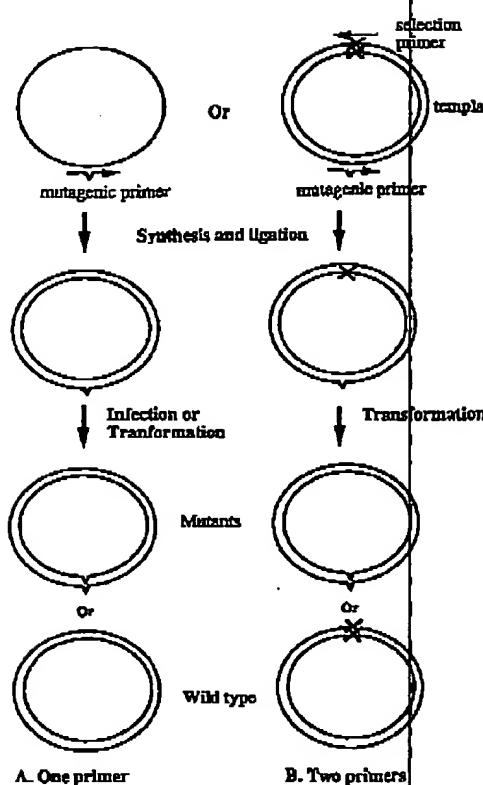
FIG. 5. The hybrid method. The mutant strand (the second strand), directed by the mutagenic primer (A, one primer) and by a selection primer (B, two primers), is synthesized using either single-stranded templates or denatured double-stranded templates under the catalysis of a thermolabile polymerase, to form a hybrid DNA carrying a wild-type strand and a mutant strand, which is then introduced into the bacteria to segregate the mutant and wild-type DNAs.

The Hybrid Method

In this classical mutagenesis method, a circular single-stranded M13 phage DNA or phagemid DNA containing the desired gene is used as the template (2, 3, 85, 86). The mutant strand (the second strand), directed by a mutagenic primer, is synthesized under the catalysis of a thermolabile DNA polymerase, such as Klenow or T4 DNA polymerase, or T7 DNA polymerase (Fig. 5A, one primer). The second strand is then sealed by T4 DNA ligase. The resulting double-stranded DNA, a hybrid carrying a wild-type strand and a mutant strand, is then introduced into an E. coli to segregate the mutant and wild-type DNAs.

Using this method, wild-type DNA background levels as high as 99.9% are often encountered after transformation, for three reasons. First, the circular single-stranded DNA template for mutant-strand synthesis is often contaminated with short DNA fragments: these

can also, like regular primers, initiate DNA synthesis, producing wild-type double-stranded DNAs that are co-transformed with the hybrid into E. coli (87). Second, the sequence in the mutagenic primer may be displaced by the strand-displacement activity of Klenow polymerase, resulting in high wild-type background (2). Third, the host-directed DNA mismatch—repair system in bacteria usually favors methylated DNAs synthesized in vivo over nonmethylated DNAs synthesized in vitro. Thus, mutations in the synthesized mutants tend to be removed within E. coli (88). It is therefore often necessary to screen a large number of clones (85).

With the introduction of selection measures (two primers), the hybrid method using single-stranded DNAs becomes more efficient. Moreover, denatured double-stranded plasmid DNAs can also be used as templates to synthesize hybrid DNAs (Fig. 5B, two primers, discussed below). However, double-stranded DNAs are less efficient than single-stranded DNAs, since there is strong competition between binding of the complementary strand and the binding of the primer to the template. This method is used mostly in the SDM.

Gapped Circles

A plasmid DNA containing a desired gene can be converted to have both single-stranded and double-stranded regions. This single-stranded region is for easy binding of a mutagenic primer. These so-called gapped circles are prepared by manipulating two DNA restriction fragments, both derived from the same plasmid. As outlined in Fig. 6, the combination of fragments I and II, of fragments I and III, or of fragments I and IV gives different pairs of gapped circles. Fragment I is made to lack the lower portion of the plasmid, which is the gene insert region, and usually is 10–40% of the whole plasmid. Fragment II is made to lack the upper portion of the plasmid and overlap with fragment I at both ends. Fragment III is a complete circular plasmid. Fragment IV is a complete linear plasmid obtained by a restriction enzyme digestion. Two fragments, in the above-mentioned combinations, are mixed, denatured, and reannealed to form a pair of gapped circles.

Depending on the designed primer, only one in each pair of gapped circles is the desired form as the template, containing a single-stranded region to which the mutagenic primer is complementary. This desired form can be gel-purified from the other form. For easier gel purification, the "gap" is better selected to be less than 12% of the total size of a plasmid (51). If a restriction site exists in the single-stranded region, the undesired gapped circle can be removed by enzyme digestion using an oligonucleotide to restore the full restriction site (12). The desired gapped circle alone can be produced by overlapping a 5'- or 3'-sticky restriction end of frag-

APPROACHES TO DNA MUTAGENESIS: AN OVERVIEW   173
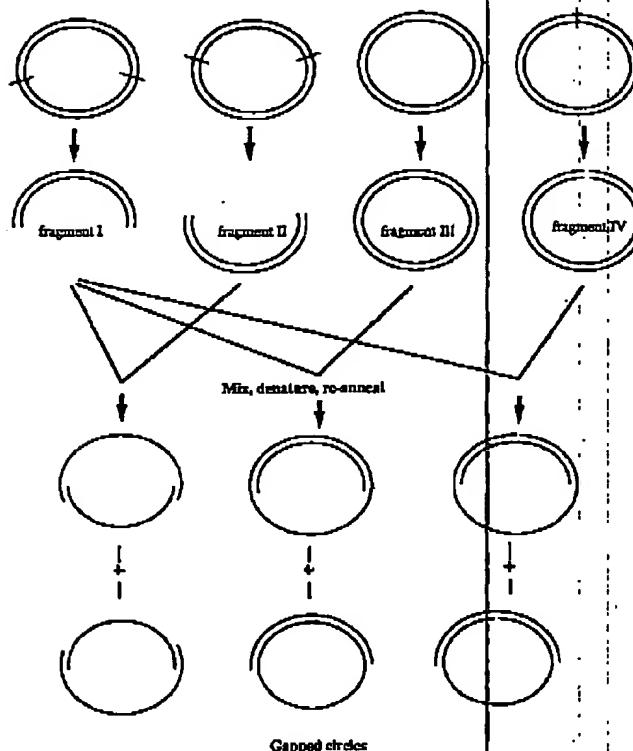
FIG. 6. The formation of gapped circles. A pair of restriction fragments is made from a plasmid; fragment I always lacks the region containing the intended mutation site. One of the other three types of fragments shown, containing the region missing in fragment I, is paired with fragment I. The two fragments in any of the three combinations shown are then mixed, denatured, and reannealed. Two forms of gapped circles are produced from each combination.

ment I with a compatible end of the second fragment (fragment II, III, or IV in Fig. 6) (52). Thus, two other strands carrying recessive restriction ends do not overlap to form the unwanted gapped circle.

The preparation of gapped circles in general is cumbersome. The purified gapped circle usually is not stable; thus, repeated preparations are unavoidable. The efficiency of mutagenesis using a gapped circle as a template with the thermolabile polymerase is relatively low, 5–15% (51); the gapped circle is no longer widely used. When certain selection measures are used together with this template (52), the efficiency can be increased (12, 53).

In Vivo Mutagenesis Methods

The "closing" oligonucleotide method. When a unique restriction site happens to be near the intended mutation position, the plasmid DNA can be cleaved at this unique site. A "closing" oligonucleotide primer

carrying mutations can be provided to cover the cleavage site. The linearized plasmid, together with this closing oligonucleotide, is cotransformed into bacteria for *in vivo* reconstitution (repairing). Only the repaired plasmid will survive in bacteria. Some of the repaired plasmids thus will contain mutations provided by closing primers (97).

The minimal length of such an oligonucleotide to seal the cleavage of a plasmid seems to be 20 nucleotides. The mutation position should be within 5 bp from the cleavage site, to obtain >15% mutagenesis efficiency. The farther the mutagenesis site from the cleavage site, the lower the efficiency. This method can be applied in the SDM.

Using bacteria with defective DNA repair systems. The error rate of DNA synthesis in a normal strain, typically 10^{-10} errors per base per generation (119), is obviously too low to be used for DNA mutagenesis. DNA repair systems individually rendered defective, e.g., *mutS* (responsible for mismatch repair), *mutD* (responsible for sequence-proofing through 3'-5' exonuclease activity), or *mutT* (responsible for hydrolyzing oxodeoxyguanosine triphosphate) (120, 121), have been shown to increase significantly the spontaneous *in vivo* mutagenesis. When *mutS, mutD*, and *mutT* were rendered defective simultaneously (122), the spontaneous mutagenesis rate in the resulting bacterial strain (XL1-Red) was increased as much as 5000-fold over that in the wild-type strain, which means one mutation occurred in a 2000-bp DNA after 12–18 h of replication. On average, every clone containing a 2-kb gene would contain a mutation, and thus a plasmid containing the gene can be introduced into a such strain to produce random mutations. Although this rate of random mutagenesis is still low compared to that of PCR-based REM methods, the simplicity of this approach is very attractive to create the REM.

Measures of Mutant Selection

In most non-PCR methods catalyzed by thermolabile DNA polymerases, a significant amount of wild-type DNA is used as templates, which often remain with mutant DNAs and are cotransformed into *E. coli.* Thus, selection measures become the key to the success of mutagenesis. To support the growth of a bacterial host in an antibiotic-containing medium, a plasmid must have a functional antibiotic resistance gene and an intact replication origin, and must be in circular form. Circular plasmids are introduced into bacteria much more efficiently than linear ones. Even if a linear plasmid is introduced into bacteria, it has to be circularized to be replicated; only about 0.1% is recircularized *in vivo*. These characteristics have become the basis for the measures of mutant selection.

174   LING AND ROBINSON

Selections Based on Restoration of a Functional Antibiotic-Resistance Gene or Replication Origin of a Mutant Plasmid

In this often-used method of selection, a plasmid in which the antibiotic-resistance gene is rendered defective (e.g., missing 1 bp) is used as a template to synthesize a hybrid plasmid, with a selection primer (a oligonucleotide to correct the defective sequence of the antibiotic-resistance gene) and a mutagenic primer (Fig. 5B, two primers) (137). As a result, only *E. coli* harboring the newly synthesized DNA carrying the selection primer sequence will survive on the antibiotic medium. To ensure that both primers are simultaneously incorporated, the mutagenic primer should be made to bind to the template preferentially. For example, its melting temperature should be higher than that of the selection primer. Both primers should also be in large molar excess (several hundred-fold) relative to template DNAs. Two resistance genes can also be used in the same plasmid, one for mutagenesis selection and the other for routine selection (138, 139). A mutagenesis efficiency of about 85% has been obtained by using this selection (138, 140).

Similarly, a selection measure can be based on the restoration of a defective replication origin (141). A phage or plasmid DNA in which the replication origin is rendered defective (e.g., missing 2 bp) can be used as a template to synthesize a hybrid plasmid in which a mutagenic primer and a selection primer (a oligonucleotide that restores the defective sequence) are incorporated into the mutant strand. Thus, only restored plasmids can be replicated in a normal *rnhA*+ strain.

Selections Based on Mutant DNAs Resistant to Restriction Digestion

Unique site elimination (USE). Wild-type plasmids containing a unique restriction site are used as templates to synthesize a hybrid plasmid with a mutagenic and a selection primer (Fig. 4B). Mismatches are introduced into this hybrid plasmid by mutagenic primers to destroy this unique restriction site. Hybrid plasmids are then transformed into an *E. coli* strain with a deficiency in correcting mismatches (*mutS*−). Plasmid DNAs extracted from pooled transformants (a mixture of wild-types and mutants) are then digested with this unique site enzyme. Since mutants do not carry this unique site, they will not be digested by this enzyme, unlike the wild-type plasmids (5, 135). The resulting indigestible plasmid is then used to re-transform a *mutS*− *E. coli* strain (11, 123, 142).

In the original method (11), approximately 2 fmol of templates was used. It has been shown subsequently that if more template (5–25 fmol) was used, more mutant clones could be made (143). Because of the increased efficiency, competent cells treated by CaCl₂

(10⁶–10⁷ cfu/mg DNA) instead of highly competent cells (10⁸–10⁹ cfu/mg DNA) may be used for the transformation. This is very useful because CaCl₂-treated competent cells are easy to prepare and use, compared to highly competent cells. If a unique site is relatively close to the desired mutagenic position, two primers, selection and mutagenic, may be combined into one long primer, which is usually made by the PCR (5, 11, 135). Although the USE method and its modifications are almost universally applicable, two rounds of transformation are required. Some restriction sites, such as *Bam*HI, *Bgl*II, and *Pvu*I, do not work well for this selection (as outlined in the booklets from the Chameleon kit, Stratagene, and the USE mutagenesis kit, Pharmacia, and experience from our lab). When the size of the plasmid is larger than 6 kb, the USE method becomes less efficient.

Digestion-resistant dNTP analogues. Mutant strands can also be distinguished by replacing one or more of the four deoxynucleotide triphosphates with their 5′-[α-thiol]triphosphate analogs during DNA synthesis. Synthesized hybrid plasmids are then treated with a restriction enzyme. Since wild-type strands contain only non-thiol sequences, they will be digested. Digested DNAs are removed by a further T7 exonuclease or exonuclease III treatment. The remaining mutant strand is then used as a template to synthesize a second mutant strand (53, 144). This selection can be used in conjunction with gapped-circle templates (53, 145, 146). A similar way of achieving this strand discrimination is to synthesize the mutant strand with methylated nucleotide triphosphate. The resulting fully methylated mutant strand will not be digested by certain frequent-cutting restriction enzymes such as *Msp*I, *Sau*3AI, and *Hha*I, but the nonmethylated wild-type strand will be digested (12, 145–147).

Selections Based on Template DNAs Susceptible to Restriction Digestion

Another powerful selection is to prepare DNA from *dut*− *ung*− bacterial strains, which do not restrict dUMP in DNAs, as do *dut*+ *ung*+ strains. Therefore, the resulting template DNA contains deoxyuracil (148). Since nascent mutant strands do not carry deoxyuracil, an ensuing digestion with the UDG enzyme exclusively eliminates template strands. Double-stranded mutant plasmids can then be made from the single-stranded mutant DNAs. Alternatively, the hybrid DNA can be transformed into a *dut*+ *ung*+ strain to eliminate the wild-type strand and to form double-stranded mutant DNAs. This selection method is often called the Kunkel method.

The template can also be prepared from a *Dam* methylase-carrying *E. coli*, which extensively methylates DNAs. The mutant strand synthesized *in vitro* is not

TABLE 3

Comparison of Major Methods of *in Vitro* Mutagenesis

	Duration (day)[a]	Man. time (h)[b]	Advantage	Disadvantage
Connection PCR[c]	6	9–10	Good efficiency, fast	Relatively shorter mutant products
Megaprimer PCR[d]	5	6–9	High efficiency, fast, larger mutant products	Needs careful setup of parameters and conditions
Inverse PCR	5	6–9	High efficiency, fast, simple, mutant product already in a plasmid	Needs to amplify long PCR products
Hybrid[e]				
Without selection	5–8	15	Mutant product already in a plasmid	Very low efficiency, lengthy, laborious,
With selection (USE)	7	12–14	High efficiency	Lengthy, laborious, some unique sites not suitable for this method
Gapped circle	7	13–15	Mutant product already in a plasmid	Relatively poor efficiency, lengthy, laborious, complex

[a] Duration is the total time from the beginning to the confirmation of mutant clones by sequencing, including waiting time (e.g., *E. coli* growth overnight).
[b] Man. time (manipulation time) is the time the operator needs to spend on performing all steps of that procedure.
[c] Connection PCR includes ligation, homologous recombination, and overlap extension of two PCR products to form the mutant product.
[d] Only the One-STEP version (19) was used for evaluation.
[e] Both methods with and without selection were evaluated. Without selection, the method needs time-consuming screening with radioactive selective hybridization (85).

methylated. When the hybrid plasmid is digested with a methylation-dependent enzyme such as DpnI, methylated wild-type template strands are removed selectively (24, 48).

Screening of Mutants

For the ease of screening of mutants made from either PCR or non-PCR methods, one of the primers should be incorporated with a sequence having a unique or rare restriction site (2, 10, 154), provided that this sequence does not bring about unwanted changes in the corresponding amino acid sequence. The desired mutations ultimately should be confirmed by sequencing. Random mutations occur even in non-PCR methods (155). For example, when T4 DNA polymerase was used in mutant DNA synthesis (6), 4 bp errors occurred in 6099 bp.

In summary of the sections on the applications of PCR and non-PCR/*in vivo* methods in DNA mutagenesis, some aspects of all major methods are compared in Table 3.

SUMMARY

In the last several years, the use of double-stranded DNA templates together with thermostable-polymerase PCR has essentially replaced the use of single-stranded DNA templates using thermolabile polymerase for *in vitro* mutagenesis. Numerous PCR methods are now available, such as overlap-extension PCR, megaprimer PCR, and inverse PCR. All of these PCR methods are reliable, effective, and convenient, al-

though they are more prone to high rates of spontaneous error in mutant DNAs than are methods using thermolabile polymerases. Some improvements, such as the introduction of methylated templates, have been employed to minimize PCR errors. On the other hand, because of the introduction of many selection measures (e.g., restoration of antibiotic resistance, restoration of replication origin and unique site elimination), both double-stranded and single-stranded DNAs can now be used as templates for mutagenesis using thermolabile polymerase methods. For PCR methods, selection measures such as nested PCR have been developed. All these selection measures have greatly improved the efficiency of mutagenesis by removing wild-type templates prior to transformation. Many efficient methods are available for both SDM and REM. Mutations can be introduced *in vitro* or *in vivo*, either by mutagenic primers or by erroneous DNA synthesis. Thus, choices largely depend on the experimental needs and resources of the investigator.

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176   LING AND ROBINSON

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APPROACHES TO DNA MUTAGENESIS: AN OVERVIEW   177

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178   LING AND ROBINSON

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